Increased Signaling via Adenosine A(1) Receptors, Sleep Deprivation, Imipramine, and Ketamine Inhibit Depressive-like Behavior via Induction of Homer1a

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Highlights

- Enhanced neuronal A_1R expression evokes antidepressant effects
- A1RKO mice display an increased depressive-like behavior and are resistant to SD
- Sihomer1a inhibits the antidepressant effects of A_1R, SD, imipramine, and ketamine
- Viral homer1a overexpression in the mPFC promotes antidepressant effects

Authors

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In Brief

The mechanism of antidepressant therapy is unclear. In this issue, Serchov et al. provide evidence for the synaptic plasticity gene homer1a as joint mechanism of pharmacological (imipramine, ketamine) and non-pharmacological (sleep deprivation) therapy in depression.

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Increased Signaling via Adenosine A_1 Receptors, Sleep Deprivation, Imipramine, and Ketamine Inhibit Depressive-like Behavior via Induction of Homer1a

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SUMMARY

Major depressive disorder is among the most commonly diagnosed disabling mental diseases. Several non-pharmacological treatments of depression upregulate adenosine concentration and/or adenosine A_1 receptors (A_1R) in the brain. To test whether enhanced A_1R signaling mediates antidepressant effects, we generated a transgenic mouse with enhanced doxycycline-regulated A_1R expression, specifically in forebrain neurons. Upregulating A_1R led to pronounced acute and chronic resilience toward depressive-like behavior in various tests. Conversely, A_1R knockout mice displayed an increased depressive-like behavior and were resistant to the antidepressant effects of sleep deprivation (SD). Various antidepressant treatments increase homer1a expression in medial prefrontal cortex (mPFC). Specific siRNA knockdown of homer1a in mPFC enhanced depressive-like behavior and prevented the antidepressant effects of A_1R upregulation, SD, imipramine, and ketamine treatment. In contrast, viral overexpression of homer1a in the mPFC had antidepressant effects. Thus, increased expression of homer1a is a final common pathway mediating the antidepressant effects of different antidepressant treatments.

INTRODUCTION

Major depression is a common disease associated with high individual suffering, increased risk of suicide, and an enormous economic burden for the society (Greenberg et al., 2003a, 2003b). Despite numerous pathophysiological hypotheses such as inadequate response to stressors resulting in alterations in neuroplasticity, neurogenesis, and neuro-immunological regulation, knowledge about the neurobiology of depression and the mechanism of action of therapeutic measures such as antidepressants, sleep deprivation (SD), and electroconvulsive therapy (ECT) are still rudimentary (Benedetti and Colombo, 2011; Kato, 2009; Krishnan and Nestler, 2010).

While the anticonvulsant, neuroprotective, and sleep-promoting effects of adenosine are widely appreciated (Fredholm et al., 2005), little is known about its potential role in mood disorders (Burnstock et al., 2011; Sadek et al., 2011; van Calker and Biber, 2005). Adenosine's actions are mediated by four receptor subtypes, A_1, A_{2A}, A_{2B}, and A_3 (Fredholm et al., 2001). It is known that SD evokes an increased release of adenosine in the brain and upregulation of adenosine A_1 receptors (A_1Rs) in rodents and humans (Basheer et al., 2007; Elmenhorst et al., 2009; Elmenhorst et al., 2007). Two other non-pharmacological interventions for depression ECT and deep brain stimulation (DBS) are associated with an increased release of adenosine and stimulation of A_1R (Bekar et al., 2008; Hamani et al., 2010; Sadek et al., 2011; van Calker and Biber, 2005). Furthermore, recent direct experimental data indicate that adenosine agonists have antidepressant activity (Hines et al., 2013). Thus, increased A1R signaling might elicit antidepressant effects.

To test directly this hypothesis, we have generated a transgenic mouse model with conditionally enhanced A_1R expression in forebrain neurons under the control of the CaMKII promoter. We report that increasing A_1R expression, by switching on the transgene in these mice, evokes both resilience against depressive-like behavior and antidepressant effects in a chronic depression model. We further show that the antidepressant effects of A_1R upregulation as well as those of SD, imipramine, and ketamine are all mediated by an induction of homer1a expression in the medial prefrontal cortex

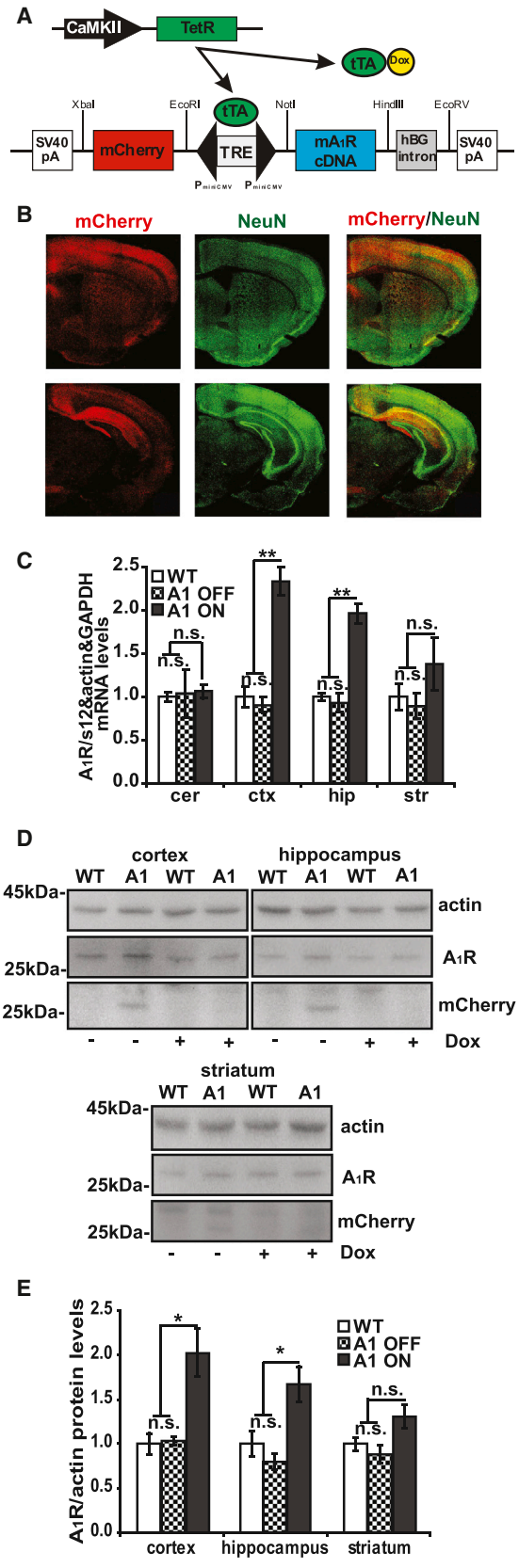

Figure 1. Generation of a Mouse Model with Tetracycline-Regulated Enhanced Expression of Adenosine A_1 Receptor Specifically in Forebrain Neurons

(A) The tetracycline-regulated (Tet-Off) expression of the mouse A_1 receptor (A_1R). Ca^{2+}/calmodulin-dependent kinase II (CaMKII) promoter controls the expression of the tetracycline transactivator (tTA) gene product, which induces the simultaneous transcription of the A_1R and mCherry specifically in forebrain neurons, by binding to a tetracycline responsive element (TRE) containing a bidirectional promoter ($P_{miniCMV}$). Thus, the gene expression could be blocked by tetracycline or its stable analog doxycycline (Dox).

(B) Spatial distribution of mCherry expression in the mouse brain (green, neuronal nuclei marker NeuN; red, anti-mCherry/RFP staining).

(C) Relative mRNA expression of A_1R in cerebellum (cer), cortex (ctx), hippocampus (hip), and striatum (str) of wild-type (WT), A1 OFF, A1 mice Dox treated from birth; A1 ON, A1 mice Dox treated from birth, followed by 4 weeks Dox withdrawal, normalized to s12, GAPDH, and actin (n = 4).

(D) Representative western blot demonstrating the effect of 4 weeks Dox treatment on mCherry and A_1R protein expression in different brain regions of WT and A1 mice (n = 4).

(E) Densitometric quantification of A_1R protein expression in different brain regions of WT and TG mice normalized to actin (n = 4).

One-way ANOVA with Bonferroni post hoc test: *$p < 0.05$, **$p < 0.01$, n.s., not significant. Data are expressed as the means ± SEM.

See also Figures S3A and S3B.

(mPFC), a neuronal immediate-early gene that has been implicated in the etiology of major depression (Rietschel et al., 2010).

RESULTS

Generation of Transgenic Mice with Inducible A_1R Expression Specifically in Forebrain Neurons

To investigate directly the behavioral effects of enhanced neuronal A_1R expression, we generated a binary transgenic mouse model with an inducible gene expression system containing a tetracycline responsive bidirectional promoter controlling the simultaneous expression of mouse A_1R and mCherry reporter gene with the Tet-OFF system (Figure 1A). The design and cloning of the tetracycline-regulated expression system was previously described and in vitro functionally characterized (Serchov et al., 2012). The generation of the A_1R transgenic mouse line was performed by pronuclear injections of the above-described construct into C57/Bl6 oocytes. To achieve region- and cell-specific upregulation of A_1R, we used CaMKII-tTA mice, which express the transactivator under the control of CaMKII promoter specifically in forebrain neurons (Mayford et al., 1996) (Figure 1A).

Evaluation of the Doxycycline-Regulated A_1R Transgene Expression

In order to prevent potential effects of enhanced transgenic A_1R expression during development, all mice were maintained on doxycycline until weaning. Thereafter, mice were either further kept on doxycycline or without doxycycline for 4 weeks to allow transgene expression. The characterization of the spatial distribution of the transgene expression, using immunohistochemical staining against the reporter gene, revealed that the mCherry expression has a labeling pattern typical for CaMKII promoter activity, primary distributed to forebrain neurons, similar to the previous reports (Mayford et al., 1996; Odeh et al., 2011)

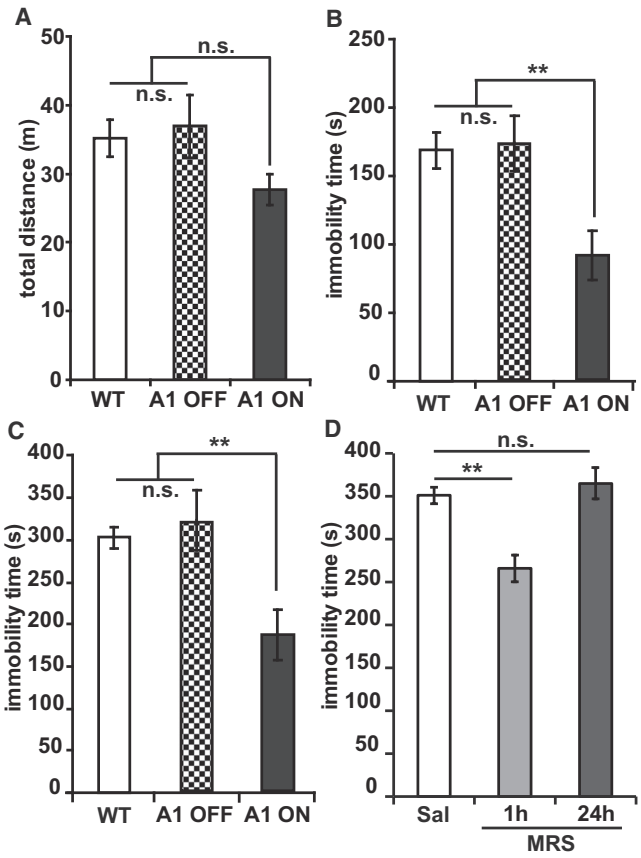

Figure 2. Antidepressant Effects of Enhanced A_1R Signaling

(A) Spontaneous activity in open field test: total distance of movement of WT and A1 mice (n = 12).

(B) Immobility time in tail suspension test (TST) (n = 8).

(C) Immobility time in day 2 of forced swim test (FST) of WT and A1 mice (n = 8).

(D) Immobility time in day 2 of FST of WT mice 1 hr and 24 hr after single i.p. injection of saline or 3 mg/kg A_1R agonist MRS5474 (n = 6).

One-way ANOVA with Bonferroni post hoc test: $^{**}p < 0.01$, n.s., not significant. Data are expressed as the means ± SEM.

See also Figure S1.

(Figure 1B). The evaluation of the doxycycline-regulated transgene expression demonstrated that doxycycline treatment completely suppressed the mCherry immunofluorescence labeling (Figure S3A) and mRNA and protein expression in the brain to undetectable levels (Figures 1D and S3B). The analysis of A_1R mRNA and protein expression levels showed about 2-fold upregulation in cortex and hippocampus in mice without doxycycline (A1 ON) in comparison to doxycycline-treated animals (A1 OFF) or their wild-type (WT) littermates (Figures 1C–1E). Low mCherry expression and no significant enhancement of A_1R mRNA and protein levels were detected in striatum and cerebellum, examined as control regions (Figures 1C–1E and S3B).

Anxiolytic and Antidepressant Effects of the Enhanced A_1R Signaling

To investigate potential effects of enhanced A_1R expression on the behavior, we subjected the mice to a battery of behavioral tests. The spontaneous activity and exploratory drive, evaluated by the total traveled distance in the open field test were not significantly affected in A1 ON mice (Figure 2A). However, switching on the transgene (A1 ON) induced robust anxiolytic effects in three well-characterized behavioral tests for anxiety: open field test (Figure S1A), elevated plus maze (Figures S1B and S1C), and dark-light box (Figures S1D and S1E). The novel object recognition test, T-maze, and Morris water maze showed that the upregulated A_1R had no significant effect on learning and memory (Figures S1F–S1K). The two basic tests for depressive-like behavior—tail suspension test (TST) and classical forced swim test (FST)—revealed marked antidepressant effects of enhanced A_1R expression, evident by significant decrease of the immobility time (Figures 2B and 2C). To test further the antidepressant effect of A_1R signaling, we intraperitoneally injected (i.p.) WT mice with the selective A_1R agonist MRS5474 (Tosh et al., 2012). MRS5474 induced a rapid antidepressant effect in FST, performed 1 hr after the injection (Figure 2D).

Antidepressant Effect of Enhanced A_1R Expression in the Chronically Despaired Mice

To investigate further the antidepressant effect of enhanced A_1R expression, we adopted the recently published chronic behavioral despair model (Kumar et al., 2010; Stone and Lin, 2011; Sun et al., 2011) (Figure 3A; see Experimental Procedures for details). In this paradigm (see Figure 3A for experimental design), WT and A1 OFF mice did not show any differences either during acquisition (induction phase) or in maintenance of depressive-like behavior (test phase) (Figures 3B and S2C). However, turning on transgenic A_1R expression after the induction phase (A1 OFF + 4 weeks ON), significantly reduced the immobility time (Figure 3B) and significantly increased travel distance (Figure S2C) to control levels, though doxycycline treatment of WT mice did not significantly affect the induction and maintenance of the depressive-like behavioral state in the chronic behavioral despair mouse model (Figures S2A and S2B). A1 ON mice exhibited pronounced resilience toward induction of the behavioral despair with significantly reduced immobility time and increased traveled distance during the induction phase but developed finally the same depressive-like behavior as A1 OFF mice at day 5 (Figures 3C and S2D). The maintenance of enhanced A_1R expression (A1 ON) or turning on transgenic A_1R expression after the induction phase (A1 OFF + 4 weeks ON) significantly reduced chronic depressive-like behavior in the test phase (Figures 3C and S2D). On the other hand, turning off transgenic A_1R expression after the induction phase (A1 ON + 4 weeks OFF) prevented this amelioration and the mice displayed depressive-like behavior indistinguishable from A1 OFF animals (Figures 3C and S2D). Similar results were obtained with TST that was performed before induction phase (TST1) and at the test phase (TST2) (see Figure 3A for experimental design). No difference in immobility time between WT and A1 OFF mice was found in both TST tests, whereas A1 ON mice displayed a significantly decreased immobility time at both time points (Figure 3D). Furthermore, after the induction phase both WT and A1 ON mice displayed decreased sucrose preference (SPT2) as compared to sucrose preferences obtained before the induction phase (SPT1) (Figure 3E). While WT mice chronically maintained

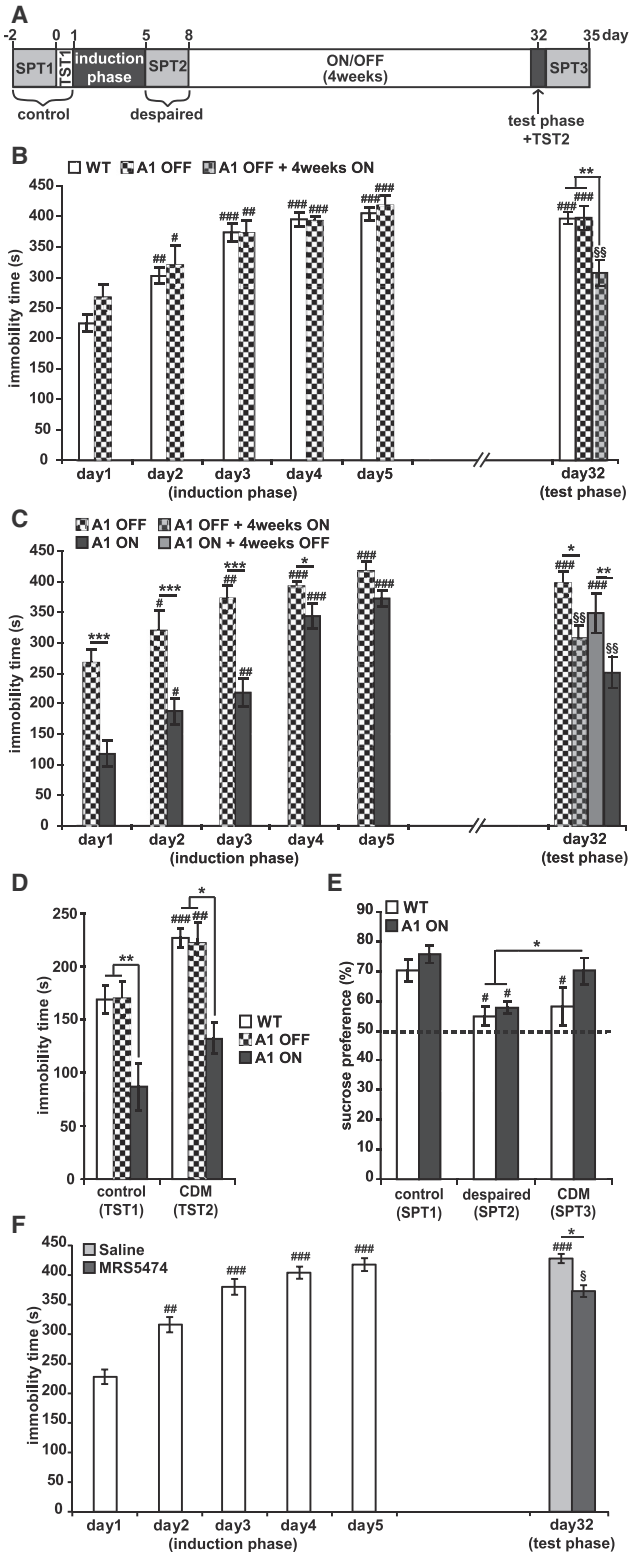

Figure 3. Antidepressant Effect of Enhanced A_1R Expression in the Chronically Despaired Mice

(A) Schematic illustration of the experimental protocol: control sucrose preference test (SPT1) and TST1 were performed at day −2 to day 0 before the induction of the behavioral despair. Then the mice were despaired by 10 min swim sessions for 5 consecutive days: day 1 to day 5 (induction phase), followed by second SPT2 (day 6 to day 8). For the following 4 weeks, the mice were kept undisturbed in their home cages and divided into five groups: WT, wild-type mice; A1 OFF, mice with doxycycline-suppressed A_1R expression for the whole experiment; A1 OFF + 4weeks ON, mice with doxycycline-suppressed A_1R expression till day 5 followed by 4 weeks of activation of the A_1R expression by doxycycline withdrawal; A1 ON, mice with activated A_1R expression for the whole experiment; A1 ON + 4weeks OFF, mice with activated A_1R expression till day 5 followed by 4 weeks suppression of the A_1R expression by doxycycline treatment. On day 32 the TST2 and the last 10 min swim session (test phase) were performed, followed by SPT3 (day 32 to day 35).

(B) Immobility time during the induction phase (day 1 to day 5) of WT (n = 20) and A1 OFF (n = 20) mice and during the test phase (day 32) of WT (n = 20), A1 OFF (n = 10) and A1 OFF + 4 weeks ON mice (n = 10).

(C) Immobility time during the induction phase (day 1 to day 5) of A1 OFF (n = 20) and A1 ON (n = 20) mice and during the test phase (day 32) of A1 OFF (n = 10), A1 OFF + 4 weeks ON (n = 10), A1 ON (n = 10), and A1 ON + 4 weeks OFF mice (n = 10). (B and C) Two-way ANOVA with Bonferroni post hoc test: *p < 0.05, **p < 0.01, ***p < 0.001; #p < 0.05, ##p < 0.01, ###p < 0.001 in comparison to day 1, and §§p < 0.01 in comparison to day 5.

(D) Immobility time in TST of control (TST1) and chronically despaired (TST2) WT (n = 10), A1 OFF (n = 10), and A1 ON (n = 10) mice. Two-way ANOVA with Bonferroni post hoc test: *p < 0.05, **p < 0.01; ##p < 0.01, and ###p < 0.001 in comparison to TST1.

(E) Sucrose preference of control (SPT1), despaired (SPT2), and chronically despaired (SPT3) WT (n = 10) and A1 ON mice (n = 10). Two-way ANOVA with Bonferroni post hoc test: *p < 0.05 and #p < 0.01 in comparison to SPT1.

(F) Immobility time during the induction phase of WT mice (n = 10) and during the test phase 1 hr after i.p. injection of saline or 3 mg/kg MRS5474. Two-way ANOVA with Bonferroni post hoc test: *p < 0.05; ##p < 0.01, ###p < 0.001 in comparison to day 1, and §p < 0.01 in comparison to day 5.

Data are expressed as the means ± SEM.

See also Figure S2.

the decreased sucrose consumption (SPT3), enhanced transgenic A_1R expression (A1 ON) for 4 weeks significantly ameliorated this anhedonic state (Figure 3E). The i.p. injection of A_1R agonist MRS5474 had a significant antidepressant effect also in chronically despaired mice, when applied 1 hr before the test phase (Figure 3F).

Behavioral Effects of A_1R Deletion

To explore further the effects of A_1R signaling, we investigated the depressive-like behavior of A1RKO mice in the chronic behavioral despair mouse model (see Figure 4A for experimental design). A1RKO mice displayed more rapid and more pronounced development of behavioral despair during the induction phase than WT mice, which was further maintained for 4 weeks, represented by significantly increased immobility time and decreased traveled distance at the induction and test phase (Figures 4B and S2E). WT mice responded to imipramine treatment (20 mg/kg/day for 4 weeks) and to 6 hr of sleep deprivation (SD) with a decrease in immobility time and increased travel distance (Figures 4B and S2E), concordant with the antidepressant effects of both treatments. Recovery sleep abolished the effects of SD on depressive-like behavior in WT animals, very similar to humans, where the antidepressant effects of SD are also lost after sleep (Figures 4B and S2E). Despaired A1RKO mice responded to imipramine treatment, but not to SD (Figures 4B and S2E). Similar results were obtained with TST (see Figure 4A

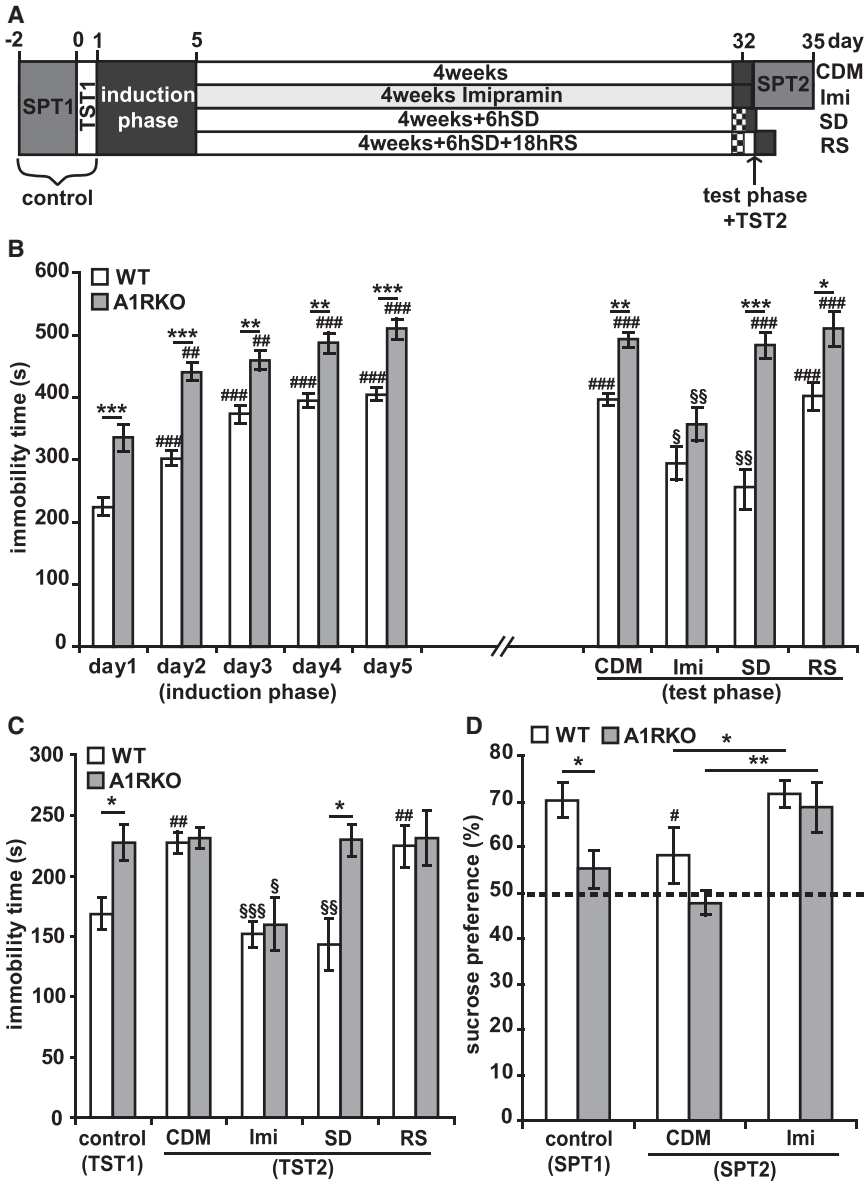

Figure 4. Behavioral Effects of A_1R Deletion

(A) Schematic illustration of the experimental protocol: control SPT1 and TST1 were performed at day −2 to day 0 before the induction of the behavioral despair. Then, the mice were despaired by 10 min swim sessions for 5 consecutive days: day 1 to day 5 (induction phase). For the following 4 weeks, the WT and A1RKO mice were kept undisturbed in their home cages and divided into four groups: CDM, chronically despaired mice; Imi, despaired mice treated for 4 weeks with Imipramine in the drinking water. SD, CDM mice sleep deprived for 6 hr on day 32. RS, CDM mice sleep deprived for 6 hr on day 32, followed by 18 hr of recovery sleep. CDM and Imi mice were subjected to TST2 and the last 10 min swim session (test phase) on day 32, followed by SPT3 (day 32 to day 35). The TST2 and test phase of SD and RS mice were performed on day 32 and day 33, respectively.

(B) Immobility time during the induction phase (n = 40) and test phase (n = 10) of WT and A1RKO mice. Two-way ANOVA with Bonferroni post hoc test: *p < 0.05, **p < 0.01, ***p < 0.001; #p < 0.05, ##p < 0.01, ###p < 0.001 in comparison to day 1 and §p < 0.05, §§p < 0.01 in comparison to day 5.

(C) Immobility time in TST of control (TST1) and CDM, Imi, SD, and RS (TST2) WT and A1RKO mice (n = 10). Two-way ANOVA with Bonferroni post hoc test: *p < 0.05, ##p < 0.01 in comparison to TST1 and §§p < 0.01, §§§p < 0.001 in comparison to CDM.

(D) Sucrose preference of control (SPT1), CDM, and Imi (SPT2) WT and A1RKO mice (n = 10). Two-way ANOVA with Bonferroni post hoc test: *p < 0.05, **p < 0.01 and #p < 0.01 in comparison to SPT1.

Data are expressed as the means ± SEM.

See also Figure S2E.

for experimental design). Also in TST1, A1RKO showed significantly increased immobility time compared to WT mice (Figure 4C). At TST2, WT animals showed reduced immobility time after imipramine treatment and SD, whereas A1RKO mice did not respond to SD with reduced immobility time in TST2, albeit the effect of imipramine was present (Figure 4C). In the sucrose preference test, A1RKO mice displayed significantly pronounced anhedonic behavior (SPT1) as compared to WT mice. Whereas sucrose intake was reduced in WT mice after chronic despair induction (SPT2), no further decrease was seen in A1RKO animals. Imipramine treatment increased sucrose intake in both A1RKO and WT animals (SPT2) to WT control levels (Figure 4D). Taken together, these results demonstrate a prominent depressive-like behavior of A1RKO mice and show that A1RKO mice are responsive to imipramine treatment, but not to SD.

Homer1a Expression Level in the mPFC Correlates with Depressive-like Behavior

Considering the potential mechanism mediating the antidepressant effects of enhanced A_1R expression, we hypothesized a role of homer1a, since this immediate-early gene product is upregulated by several antidepressant treatments (Conti et al., 2007; Maret et al., 2007; Sakagami et al., 2005; Sun et al., 2011) and was recently implicated in the etiology of major depression (Rietschel et al., 2010). Thus, we initially checked homer1a mRNA expression by in situ hybridization (Figures 5A and S3C) and qRT-PCR (Figures 5C and S3D) in different brain regions of WT and A1 ON mice. We found significantly increased homer1a mRNA levels in the cortex, including mPFC and hippocampus, but not in the striatum of A1 ON mice (Figures 5A, 5C, S3C, and S3D), regions in which enhanced A_1R expression was detected (Figures 1C–1E). Since in situ hybridization data (Figures 5A and S3C) matched the results obtained by qRT-PCR (Figures 5C and S3D), we further used qRT-PCR to evaluate homer1a mRNA expression. Then Homer1a mRNA expression was analyzed in

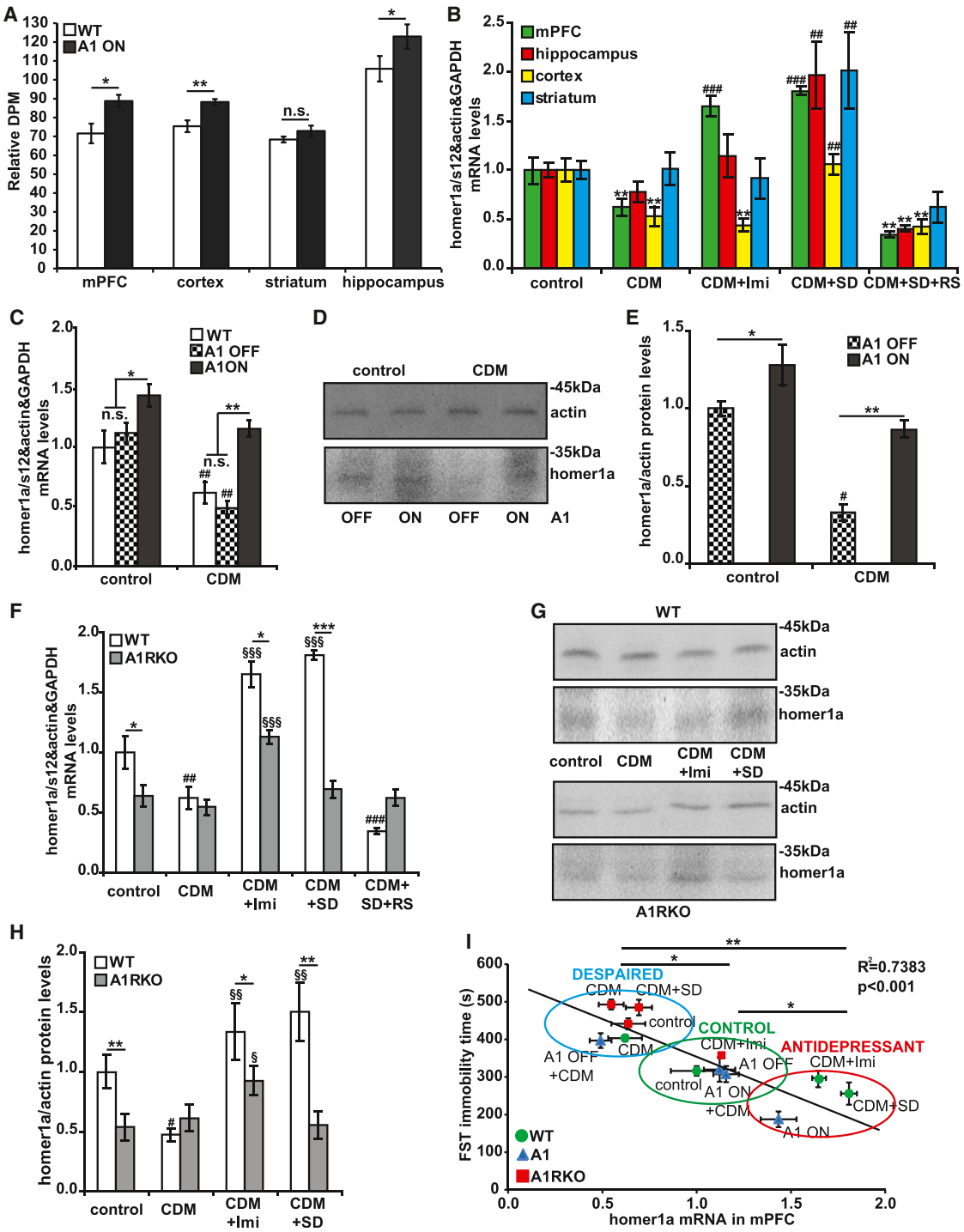

Figure 5. Homer1a Expression Level in Medial Prefrontal Cortex Correlates with Mouse Depressive-like Behavior

(A) Densitometric quantifications of homer1a mRNA expression levels determined by in situ hybridization in medial prefrontal cortex (mPFC), cortex, striatum, and hippocampus of WT and A1 ON mice (n = 4). Student's t test: *p < 0.05, **p < 0.01, n.s., not significant.

(B) qRT-PCR analysis of relative homer1a mRNA expression levels in mPFC, hippocampus, cortex, and striatum of control, chronically despaired (CDM), despaired mice treated for 4 weeks with Imipramine (Imi), despaired mice sleep deprived for 6 hr (SD), and despaired mice sleep deprived for 6 hr followed by 18 hr recovery sleep (RS) (n = 6). One-way ANOVA with Bonferroni post hoc test: **p < 0.01 in comparison to control; ##p < 0.01, ###p < 0.001 in comparison to CDM.

(C) Relative homer1a mRNA expression levels in the mPFC of control and CDM WT, A1 OFF, and A1 ON mice (n = 6).

(D) Representative western blot of homer1a protein expression in mPFC of control and CDM A1 OFF and A1 ON mice.

(legend continued on next page)

WT animals subjected to the chronic despair paradigm with and without imipramine treatment or SD. Mice in the state of chronic despair (CDM) showed reduced homer1a mRNA expression in the cortex (mPFC and cortex), but not in hippocampus and striatum (Figure 5B). Imipramine treatment only increased homer1a mRNA expression in the mPFC, whereas SD led to homer1a increase in all examined brain areas (Figure 5B). Thus, homer1a expression only in the mPFC was inversely correlated to the depressive-like behavior of WT animals. We therefore further examined the homer1a levels in transgenic and A1RKO in this region. We found an increased homer1a mRNA (Figures 5A and 5C) and protein (Figures 5D and 5E) expression in the mPFC of A1 ON as compared to WT and A1 OFF mice under control conditions or when mice were subjected to the chronic despair paradigm. In contrast, A1RKO mice displayed decreased homer1a mRNA expression in all investigated regions (Figures 5F and S3D). A1RKO mice, which were resistant to the antidepressant effects of SD, displayed no significant induction of homer1a mRNA and protein levels in mPFC after SD (Figures 5F–5H). This lack of effect on homer1a expression was specific since another neuronal immediate-early gene Arc was efficiently upregulated in mPFC (Conti et al., 2007) (Figure S3G), indicating that A1RKO mice are able to respond to SD. Homer1a expression in mPFC was strongly upregulated by imipramine treatment in both WT and A1RKO mice (Figures 5F–5H). In contrast, the expression of the long splice variant homer1b/c, measured as control, was not significantly affected, either by the genotype or the treatment (Figures S3E and S3F).

Taken together, these data indicate a significant inverse correlation between mouse depressive-like behavior and homer1a mRNA expression in the mPFC (Figure 5I). The mice can be divided into three significantly different groups, according to these factors: "DESPAIRED" with high immobility time and low homer1a expression; "CONTROL" with moderate immobility time and homer1a expression; and "ANTIDEPRESSANT" with low immobility time and high homer1a expression (Figure 5I).

A_1R Signaling Induces Homer1a Expression in Primary Neuronal Cultures and Mouse Cortex

As homer1a mRNA expression was increased in all investigated brain regions of A1 ON mice, where enhanced A_1R expression was detected (Figures 5A, S3C, and S3D), we asked whether A_1R stimulation leads to homer1a induction. Indeed, we found that the adenosine receptors agonist NECA rapidly but transiently upregulated homer1a mRNA (Figure 6A) and protein (Figures 6C, 6D, and S3I) in cultured primary neurons. This homer1a induction was absent in primary neurons from A1RKO mice (Figures 6B–6D), indicating the involvement of A_1R. As a positive control, NMDA stimulation similarly induced homer1a mRNA expression in the cultures from both strains (Figures 6A and 6B). No effect of NECA was found on homer1b/c levels, measured as control (Figure S3H). We investigated further the A_1R-dependent increase of homer1a expression by applying several different inhibitors of A_1R signaling. Thus, we pretreated neuronal cultures for 30 min with G protein blocker pertussis toxin (10 ng/ml; PTX), PLC inhibitor U37122 (50 μM), or ERK1,2 inhibitor PD98058 (30 μM) prior to the NECA stimulation. These inhibitors completely abolished the NECA-mediated increase of homer1a mRNA expression (Figure 6E), indicating that the ERK-pathway is involved in the homer1a upregulation. Indeed, in vivo i.p. application of of A_1R agonist MRS5474 (3 mg/kg) strongly increased ERK1,2 phosphorylation (Figure 6F) and significantly induced homer1a protein expression (Figure 6G) in the mouse cortex after 1 hr.

SiRNA Knockdown of Homer1a Expression in mPFC Inhibits the Antidepressant Effects of Enhanced A_1R Expression, SD, Imipramine, and Ketamine Treatment

To investigate whether the action of antidepressant treatments is due to the increased homer1a expression, we knocked down the homer1a mRNA specifically in the mPFC using custom-designed anti-homer1a Accell siRNA (see the Supplemental Experimental Procedures in the Supplemental Information and Figure S8 for more details about siRNA design and in vitro testing of the efficacy). SiRNA downregulation of Homer1a in the mPFC (Figure 7E) significantly increased the depressive-like behavior of WT mice and inhibited the antidepressant effect of enhanced A_1R expression in A1 ON mice (Figure 7B). However, sihomer1a had no significant effect on the spontaneous locomotor activity (Figure 7A) and anxiety-like behavior (Figure S7C) of the mice in open field test.

To check whether homer1a is necessary for the antidepressant effect of SD, we injected sihomer1a in the mPFC of chronically despaired WT mice 2 days prior to the SD and the test phase (Figure 7C). Since chronically despaired mice had already low homer1a expression in the mPFC, compared

(E) Densitometric quantification of homer1a protein expression levels in mPFC of control and CDM A1 OFF and A1 ON mice normalized to actin (n = 4).
(F) Relative homer1a mRNA expression levels in the mPFC of control, CDM, Imi, SD, and RS WT and A1RKO mice (n = 6).
(G) Representative western blot of homer1a protein expression in mPFC of control, CDM, Imi, and SD WT and A1RKO mice.
(H) Densitometric quantification of homer1a protein expression levels in mPFC of control, CDM, Imi, and SD WT and A1RKO mice normalized to actin (n = 4). Two-way ANOVA with Bonferroni post hoc test: *p < 0.05, **p < 0.01; #p < 0.05, ##p < 0.01, ###p < 0.001 in comparison to control and §p < 0.05, §§p < 0.01, §§§p < 0.001 in comparison to CDM.
(I) Homer1a mRNA level in mPFC correlates with mouse depressive-like behavior. The linear regression line represents the significant correlation between homer1a mRNA relative expression in mPFC and the immobility time spent in day 2 of FST or test phase (Pearson product-moment correlation coefficient R^2 = 0.7383; p < 0.001). The mice formed three significantly different groups: DESPAIRED (blue circled) with high immobility time and low homer1a expression (control A1RKO [control] and chronically despaired [CDM] WT, A1 OFF, A1RKO, and sleep-deprived A1RKO [CDM+SD] mice); CONTROL (green circled) with middle levels of immobility time and homer1a expression (control WT and A1 OFF, and chronically despaired A1 ON and A1RKO treated with imipramine (CDM+Imi) mice); ANTIDEPRESSANT (red circled) with low immobility time and high homer1a expression (control A1 ON and chronically despaired WT mice treated with imipramine or SD). Two-way ANOVA with Bonferroni post hoc test: *p < 0.05, **p < 0.01.
Data are expressed as the means ± SEM.
See also Figures S3C–S3G and S4 and Table S1.

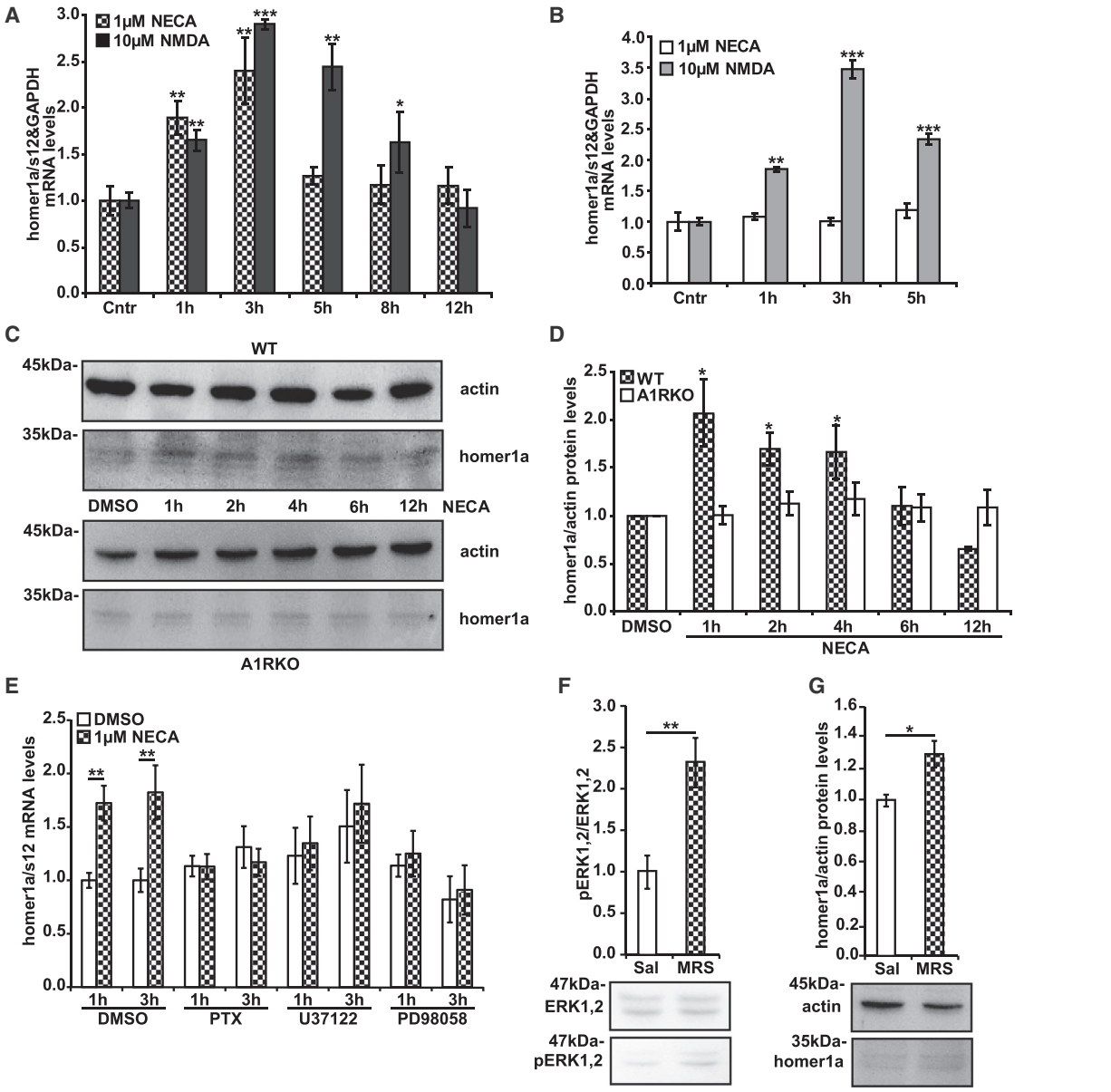

Figure 6. A_1R Signaling Induces Homer1a Expression in Primary Neuronal Cultures and Mouse Cortex

(A) Relative homer1a mRNA expression levels in WT primary neurons stimulated with 1 μM NECA or 10 μM NMDA for indicated time periods (n = 4).

(B) Relative homer1a mRNA expression levels in A1RKO primary neurons stimulated with 1 μM NECA or 10 μM NMDA for indicated time periods (n = 3).

(C) Representative western blots of homer1a protein expression in 1 μM NECA stimulated primary neurons from WT and A1RKO mice.

(D) Densitometric quantification of homer1a protein expression in 1 μM NECA stimulated primary neurons from WT and A1RKO mice (n = 3).

(E) Relative homer1a mRNA expression levels in WT primary neurons pretreated 30 min, prior to 1 μM NECA stimulation, with different inhibitors of A_1R signaling cascade (10 ng/ml PTX, 50 μM U37122, and 30 μM PD98058) for indicated time periods (n = 3).

One-way ANOVA with Bonferroni post hoc test: *p < 0.05, **p < 0.01, ***p < 0.001 in comparison to Cntr.

(F and G) Representative western blots and densitometric quantifications of ERK1,2 phosphorylation (F) and homer1a protein expression level (G) in the cortex of mice 1 hr after i.p. injection of saline (Sal) or 3 mg/kg A_1R agonist MRS5474 (MRS) (n = 3). Student's t test: *p < 0.05, **p < 0.01.

Data are expressed as the means ± SEM.

See also Figures S3H, S3I, and S4 and Table S1.

to naive controls (Figures 5), the further downregulation of homer1a by siRNA (Figure 7E) did not affect the immobility time of the animals during the test phase (Figure 7D). However, sihomer1a injections completely inhibited both the homer1a induction (Figure 7E) and the antidepressant effect of 6 hr SD (Figure 7D).

We further tested whether homer1a downregulation in the mPFC inhibits the antidepressant effects of imipramine and

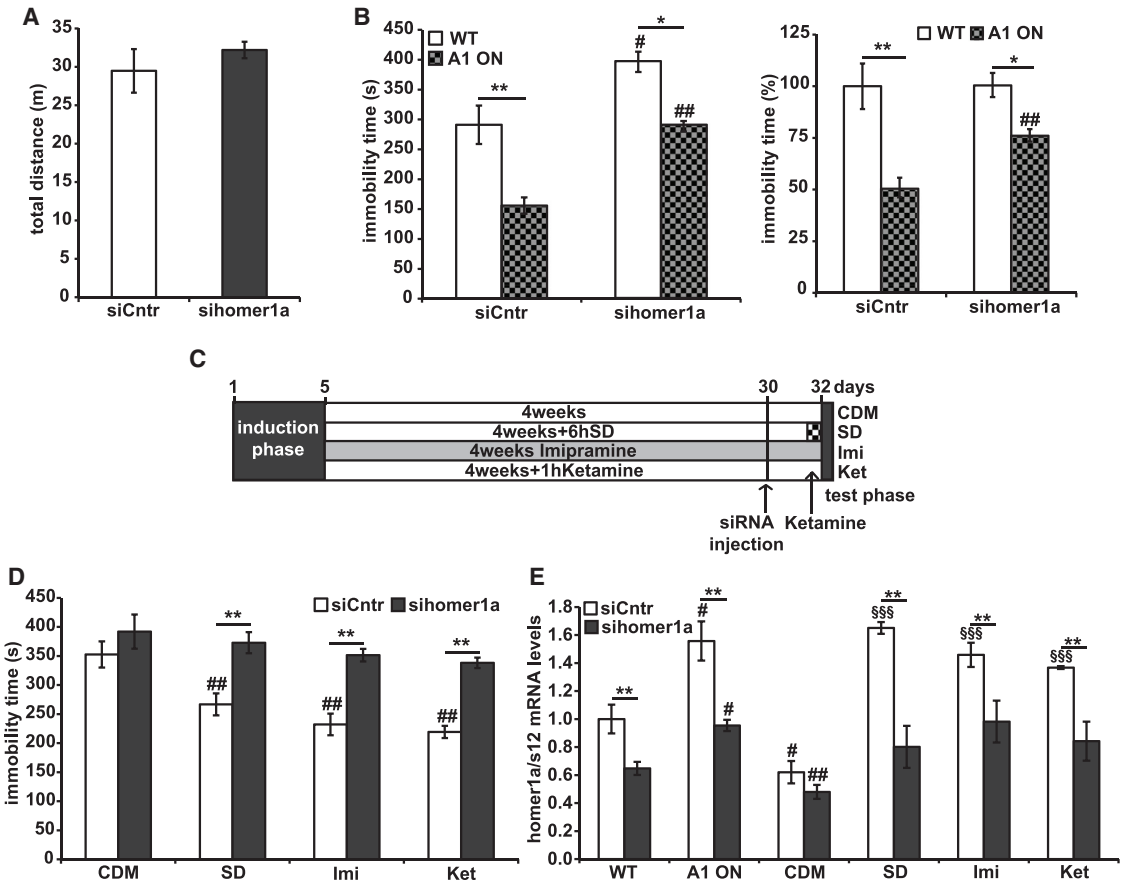

Figure 7. Knockdown of Homer1a Expression with siRNA in mPFC Inhibits the Antidepressant Effects of Enhanced A_1R Expression, Sleep Deprivation, Imipramine, and Ketamine Treatment

(A) Spontaneous activity in open field test of WT mice stereotaxically bilaterally injected with anti-homer1a (sihomer1a) (n = 7) or non-target control (siCntr) (n = 7) siRNA into mPFC.

(B) Immobility time during day 2 of FST of WT (n = 14) and A1 ON (n = 12) mice plotted in seconds (left) and as percentage of WT (right). The animals were stereotaxically bilaterally injected with anti-homer1a (sihomer1a) or non-target control (siCntr) siRNA into mPFC and 2 days later subjected to FST. Two-way ANOVA with Bonferroni post hoc test: *p < 0.05, **p < 0.01; #p < 0.05, ##p < 0.01 in comparison to siCntr.

(C) Schematic illustration of the experimental protocol. WT mice were despaired by 10 min swim sessions for 5 consecutive days (day 1 to day 5). The mice were kept undisturbed in their home cages for 4 weeks and divided into four groups: CDM, chronically despaired mice; SD, CDM subjected to 6 hr SD; Imi, CDM treated with imipramine I the drinking water for 4 weeks; Ket, CDM treated with ketamine 1 hr before the test phase. On day 30 the mice were siRNA injected into mPFC and the last 10 min swim session (test phase) was performed on day 32.

(D) Immobility time during the test phase of CDM, SD, Imi, and Ket mice injected with siCntr or sihomer1a into mPFC (n = 12). Two-way ANOVA with Bonferroni post hoc test: **p < 0.01; ##p < 0.01 in comparison to CDM.

(E) Relative homer1a mRNA expression levels in mPFC of WT, A1 ON, CDM, SD, Imi, and Ket mice injected with siCntr or sihomer1a into mPFC (n = 12). Two-way ANOVA with Bonferroni post hoc test: **p < 0.01; #p < 0.05, ##p < 0.01 in comparison to WT, §§§p < 0.001 in comparison to CDM.

Data are expressed as the means ± SEM.

See also Figures S5, S6, and S7.

ketamine. While at least 2 weeks of imipramine (20 mg/kg/day) treatment is necessary to promote antidepressant effects in CDM mice (Figure S5A) and induce homer1a mRNA levels in the mPFC of chronically despaired mice (Figure S5C), only a single i.p. injection of ketamine (3 mg/kg) was sufficient to promote rapid and long-lasting antidepressant effects (Figure S5B) and sustained significant increase of homer1a mRNA expression in the mPFC (Figure S5C). Sihomer1a injections into mPFC significantly suppressed both the homer1a induction (Figure 7E) and the antidepressant effects of chronic imipramine and acute ketamine treatment (Figure 7D). Taken together, these data point toward a general importance of homer1a for anti-depressive therapy.

Viral Overexpression of Homer1a in mPFC Promotes Antidepressant Effects in Chronically Despaired Mice

To address directly the role of increased homer1a expression on the mouse depressive-like behavior, we used recombinant adeno-associated viral vectors (rAAVs) carrying Homer1a (h1aV), mutated Homer1aW24A (h1aV(W24A)), which is unable to bind

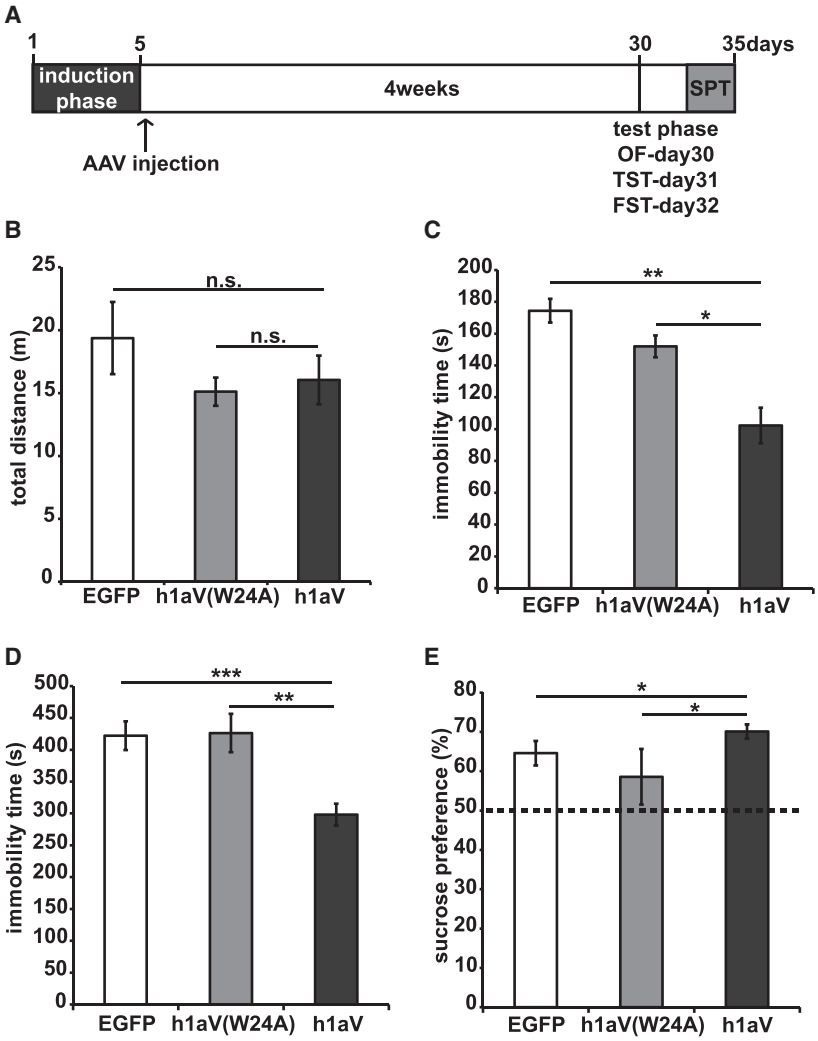

Figure 8. Intra-mPFC Injection of rAAV-Homer1a Promotes Antidepressant Effects in Chronically Despaired Mice

(A) Schematic illustration of the experimental protocol: WT mice were despaired by 10 min swim sessions for 5 consecutive days (day 1 to day 5) followed by stereotaxical bilateral injection of rAAV into mPFC. The mice were kept undisturbed in their home cages for 4 weeks and then subjected to open field test (OF) (day 30), TST (day 31), FST (day 32), and SPT (day 32 to day 35).

(B) Spontaneous activity in OF: total distance of movement of CDM WT mice injected with of rAAV-EGFP (EGFP) (n = 5), rAAV-Homer1aVenus(W24A) (h1aV(W24A)) (n = 4), or rAAV-Homer1aVenus (h1aV) (n = 10) into mPFC.

(C) Immobility time in TST.

(D) Immobility time in FST.

(E) Sucrose preference in SPT.

One-way ANOVA with Bonferroni post hoc test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Data are expressed as the means ± SEM.

See also Figure S7.

proline-rich motifs, and EGFP cDNA, used as control (Lominac et al., 2005). Chronically despaired WT mice were stereotaxically bilaterally injected with rAAV into mPFC on day 6 immediately after the training phase (Figure 8A) and behaviorally tested 4 weeks later. rAAV-mediated overexpression of homer1a did not affect the spontaneous locomotor activity (Figure 8B) and the anxiety-like behavior (Figure S7D) of the mice in open field test. However, homer1a-injected mice showed strongly reduced depression-like behavior in TST (Figure 8C) and FST (Figure 8D) and significantly increased sucrose consumption in SPT (Figure 8E), in comparison to the control mice expressing mutated h1aV(W24A) or EGFP.

DISCUSSION

Numerous studies have suggested a potential role of adenosinergic signaling in the mechanism of action of SD, ECT, and DBS (Bekar et al., 2008; Hamani et al., 2010; Hines et al., 2013; Sadek et al., 2011; van Calker and Biber, 2005). To analyze directly the potential antidepressant effects of increased A_1R signaling, we have created a transgenic mouse model with conditioned upregulation of A_1R selectively in forebrain neurons. In order to maximize the specificity, we used the forebrain neuron-specific CaMKII promoter and Tet-OFF system, avoiding potential artifacts of the transgene during development. The transgene expression in our mouse model generated levels of A_1R upregulation comparable to what is known in animal and human brain in response to seizures or sleep deprivation (Biber et al., 2008; Elmenhorst et al., 2007, 2009; Vanore et al., 2001).

Our behavioral analyses demonstrated that upregulation of A_1R caused a robust anxiolytic effect but did not affect spontaneous activity, exploratory behavior, spatial learning, or recognition and working memory. These results are in line with findings published using A1RKO mice, which demonstrate increased anxiety-like behavior without affecting motor performance, exploration, or learning and memory (Giménez-Llort et al., 2002; Johansson et al., 2001; Lang et al., 2003). Moreover, our data corroborate the importance of A_1R in controlling anxiety behavior in both rodents (Prediger et al., 2006) and humans, as it was published recently that individuals with increased anxiety also show changes in A_1R binding in vivo (Hohoff et al., 2014).

The two basic tests for depressive-like behavior TST and the classical FST revealed significant antidepressant effects of the enhanced A_1R expression. We further validated the antidepressant effects of upregulated A_1R in an experimental model of chronic depression that causes a chronic state of behavioral despair for at least 4 weeks (Kumar et al., 2010; Stone and Lin, 2011; Sun et al., 2011). The chronic maintenance of the depression-like state in this model was confirmed by TST and by SPT. The time frame of 4 weeks, between the induction and test phase, offered the possibility of therapeutic intervention.

Inducing A_1R expression in our model reduced the immobility time and increased the sucrose preference similarly to the antidepressant effects of 4 weeks treatment with imipramine or 6 hr of SD. Thus, the enhanced A_1R expression not only provides protective antidepressant effect against the development of depressive-like behavior, but also elicits "therapeutic" effects in mice with already induced chronic depression-like behavioral symptoms.

On the other hand, A_1R deficiency sensitizes to depressive-like behavior and obviates the antidepressant-like response to SD. Thus, it is concluded that A_1R are crucial for the antidepressant effects of SD. These results confirm and extend recent findings showing that the SD-induced antidepressant effects are due to adenosine release from astrocytes, which acts via adenosine A_1R presumably expressed on neurons (Hines et al., 2013). However, the observed increased depression-like behavior of A1RKO mice in our experiments differed from previously published studies using another A1RKO mouse strain (Hines et al., 2013; Sun et al., 2001). Indeed, other behavioral differences between the two A1RKO mouse lines were reported previously, which might be the result of distinct genetic background or different testing conditions (Giménez-Llort et al., 2002; Johansson et al., 2001; Lang et al., 2003).

Furthermore, we describe here an antidepressant effect of the selective A_1R agonist MRS5474 (Tosh et al., 2012). MRS5474 has been recently developed as a potent selective A_1R agonist that is active in the CNS upon peripheral administration without cardiovascular side effects. This allows i.p application and tolerability over a wide dose range, unlike all other known A_1R agonists, and it has less effect on the locomotor activity of the animals (Tosh et al., 2012). These data not only corroborate the crucial role of A_1R in depressive-like behavior but point toward potential new therapeutic possibilities of peripheral application of A_1R agonists. MRS5474 had a rapid and relatively short-term antidepressant effect in both classical FST and chronically despaired mice. Likewise, the antidepressant effects promoted by SD and i.c.v. treatment with A_1R agonists are only transient (Hines et al., 2013), suggesting that A_1R is rapidly desensitized. Our data also show that if desensitization is counterbalanced by transgenic expression, as in our mouse model, the antidepressant effects of A_1R persist. Thus, the enhanced A_1R function is directly coupled to the antidepressant effect.

As one possible mechanism mediating the antidepressant effects of enhanced A_1R expression, we considered a role of homer1a. A potential involvement of homer1a in depression-like behavior has been suggested in earlier reports (Kato, 2009; Lominac et al., 2005; Rietschel et al., 2010; Sakagami et al., 2005; Sun et al., 2011; Szumlinski et al., 2006). Interestingly, homer1a is upregulated by SD and ECT, antidepressant therapies also associated with increased A_1R signaling (Conti et al., 2007; Elmenhorst et al., 2007, 2009; Maret et al., 2007; Sadek et al., 2011; Sakagami et al., 2005). The investigation of the homer1a levels in A1 ON and A1RKO mice in combination with our in vitro studies clearly demonstrates that A_1R regulates homer1a expression. A_1R are usually coupled with Gi proteins, which inhibit cAMP formation (Fredholm et al., 2001), but when highly expressed in cells (for example, neurons or smooth muscle cells), A_1R can also regulate phospholipase C (Biber et al., 1997; Fenton et al., 2010; Robin et al., 2011; Rogel et al., 2006) and MAPK pathway (Kunduri et al., 2013; Migita et al., 2008). Indeed, blocking this pathway completely abolished the NECA-mediated increase of homer1a expression in primary neurons. In addition, MRS5474 induces ERK1,2 activation and homer1a levels in the mouse cortex, corroborating previous reports on ERKs in homer1a regulation (Mahan et al., 2012; Sato et al., 2001) and providing evidence for the importance of this signaling route in the normal brain.

The molecular mechanism of SD is poorly understood, but the potential role of several immediate-early genes, including Arc and homer1a, has been suggested (Benedetti and Colombo, 2011; Conti et al., 2007; Grønli et al., 2013; Maret et al., 2007). We have shown here that the A1RKO mice are resistant to the antidepressant effect of SD and show no significant induction of homer1a levels in the mPFC after SD. In contrast, Arc expression was increased in A1RKO (Figure S3G), serving as positive control. Thus, the antidepressant effects of SD appear to be due to the A_1R-mediated induction of homer1a.

We have found that the antidepressant effects mediated by the enhanced A_1R expression, SD, imipramine, and ketamine treatment are all accompanied by and strictly dependent on an increased expression of homer1a specifically in the mPFC. We show here that in vivo anti-homer1a Accell siRNA injections (Nakajima et al., 2012) decreased homer1a mRNA in the mPFC to levels similar to that found in chronically despaired mice. Indeed, this downregulation increased the depressive-like behavior of WT animals and obviated the antidepressant effects of enhanced A_1R signaling (A1 ON mice and in WT animals after SD). The siRNA effect was more obvious in the SD experiment as here CDM mice were used that express low levels of homer1a. Homer1a was also found upregulated by treatment with classical antidepressants, such as imipramine and fluoxetine, as well as ketamine, a compound producing very rapid and sustained antidepressant effects also in humans (Browne and Lucki, 2013), extending preliminary results by others (Conti et al., 2007; Serres et al., 2012; Sun et al., 2011). Sihomer1a application into mPFC inhibited homer1a induction and prevented the antidepressant effects caused by chronic imipramine and acute ketamine treatment. Indeed, depressive-like behavior correlated significantly with homer1a mRNA expression in the mPFC (Figure 5I).

How ketamine or imipramine treatment leads to enhanced homer1a expression in the mPFC is unclear but direct effects of both drugs in the mPFC seem unlikely. Ketamine as NMDA receptor antagonist may not have a direct effect on homer1a as NMDA receptors usually are inducers of homer1a in neurons. Accordingly, studies that show direct effects of ketamine on Homer1a in neurons are currently not available. The fact that in CDM mice imipramine needs to be given for at least 2 weeks to increase Homer1a argues against a direct effect of imipramine in the mPFC. We hypothesize that ketamine and imipramine change the network activity of the brain and that as a common result of those changes Homer1a in the mPFC is upregulated. Indeed, changes in brain network activity are discussed as instrumental for depression and its treatment (Voytek and Knight, 2015).

Importantly, viral overexpression of homer1a in the mPFC promotes antidepressant effects in chronically despaired mice in

several behavioral tests. These observations extend further the earlier findings describing that overexpression of homer1a ameliorated the increased depression-like behavior of homer1 KO mice (Lominac et al., 2005). While some reports suggested that homer1a is implicated in several neuropsychiatric disorders (Szumlinski et al., 2006), we demonstrated here that changes of homer1a levels in the mPFC has no significant effect on the anxiety-like behavior, spontaneous activity, and exploratory drive (Figure S7).

How homer1a mediates antidepressant effects is currently not understood. The long homer scaffolds are bridging metabotropic glutamate receptors with many proteins involved in Ca^{2+} signaling (Fagni et al., 2000; Jardin et al., 2013), which have been implicated in the pathophysiology of mood disorders (Galeotti et al., 2008a, 2008b). The short homer1a is lacking the carboxyl-terminal domain and is considered as a dominant-negative regulator of these interactions by disrupting homer clusters by competitive binding to target proteins (Kammermeier and Worley, 2007; Shiraishi-Yamaguchi and Furuichi, 2007). Its general role is thought to involve an activity-dependent synaptic reorganization (Hu et al., 2010; Inoue et al., 2007) that provides flexible adaptation to environmental demands. Thus, as much as clinical depression can be seen as the result of failed adaptation to stress, homer1a upregulation might evoke its antidepressant effects by improving synaptic reorganization in neural networks salient for mood regulation. Indeed, homer1a has been identified as a member of the hitherto enigmatic group of plasticity-related proteins that promote synaptic reorganization by stabilizing "tagged" synapses (Okada et al., 2009; Worley and Shuler, 2014), as predicted by the "tagging and capture" hypothesis of memory formation (Redondo and Morris, 2011).

Taken together, enhanced A_1R expression (in transgenic mice or induced by SD) provides protection against acquisition of depressive-like behavior and therapeutic effects when depression-like behavior is already induced, both mediated by homer1a upregulation in the mPFC. It is suggested that similar mechanisms are also present in antidepressant therapies used in patients, like ECT, DBS, or transcranial magnetic stimulation, where enhanced A_1R signaling has been reported (Bekar et al., 2008; Hamani et al., 2010; Kato, 2009; Sakagami et al., 2005).

Since homer1a is upregulated by several different antidepressant treatments, including those with a very rapid effect like ketamine, it is concluded that homer1a induction is a crucial joint mechanism mediating the antidepressant effects. As in humans, homer1 gene variants have been associated with the etiology of major depression (Rietschel et al., 2010) our findings provide potential insights into the general mechanism of antidepressant therapy.

EXPERIMENTAL PROCEDURES

See the Supplemental Experimental Procedures in the Supplemental Information for more details.

Mice

All procedures were performed in accordance with the German animal protection law, FELASA, the national animal welfare body GV-SOLAS, and NIH guide for the care and use of laboratory animals and were approved by the animal welfare committee of the University of Freiburg, University of Bonn, University of Naples, and NIH. The adenosine A_1 receptor knockout mouse line (A1RKO) used in this study was previously described (Sun et al., 2001).

Behavioral Studies

Activity and behavior of mice were observed using an automatic video-tracking system for recording and analysis (VideoMot2 system V6.01, TSE), unless otherwise specified. One cohort of mice was used to perform the open field, object recognition test, light/dark transition test, T-maze, elevated plus maze, and Morris water maze and another cohort was used for the classical tail suspension and forced swim tests. The chronic behavioral despair induced by repeated swim stress and sucrose preference test was performed with both cohorts.

Chronic Behavioral Despair Model

In order to induce chronic behavioral despair in mice, we used a recently described protocol (Sun et al., 2011). The mice were subjected to repeat swimming in a transparent cylinder (15 cm diameter) containing 20 cm of water (22–25°C) for 10 min daily for 5 consecutive days (induction phase). From day 6 on, the mice were kept in the home cage without swimming for 4 weeks, after which a last swim was imposed on day 32 (test phase). The immobility time and the swum distance of the mice were analyzed in each session. The repeated exposure to swimming significantly increased the immobility time and decreased the traveled distance over this 5 day period. This induced state of behavioral despair was chronically maintained for 4 weeks (till day 32) and represent a model for depressive-like behavior in mice (Sun et al., 2011).

Other Behavioral Studies, Immunohistochemistry, In Situ Hybridization, Quantitative Real-Time PCR, Western Blot Analysis, Primary Neuronal Cultures, and In Vitro siRNA Interference

See the Supplemental Experimental Procedures.

In Vivo Stereotaxic Microinjections of siRNA and Recombinant Adeno-Associated Viral Vectors

Endotoxin-free FAM-labeled Accell Green non-targeting control siRNA and the in vitro tested si2: 5′-CAGCAATCATGATTAAGTA-3′ (Moutin et al., 2012) anti-homer1a Accell siRNA (Thermo Fisher Scientific) (Nakajima et al., 2012) (2 μg/μl) dissolved in Accell siRNA delivery media (Thermo Fisher Scientific) were stereotaxically bilaterally injected with a Hamilton syringe fitted with a 33G needle aimed above the mPFC (anteroposterior +1.34 mm, mediolateral ± 0.6 mm, dorsoventral −2.0 mm, relative to bregma) (Lominac et al., 2005) at a rate of 0.1 μl/min for 5 min (total volume of 0.5 μl/side). The injection needle was shortly left in place and slowly withdrawn (1 mm/min) after the injection. Afterward, animals were allowed to recover and treated for 24 hr with Metamizole (200 mg/kg daily dose) (Zentiva) in drinking water. Behavioral testing commenced 2 days after the injection. The siRNA transfection efficacy and localization was verified by qRT-PCR and immunohistochemistry.

For stereotaxic injections of rAAVs (Celikel et al., 2007), 1.5 μl of either, rAAV-EGFP, -h1aV, or -h1aV(W24A) (~2 × 1,011 particles/ml) were injected bilaterally into the mPFC (Lominac et al., 2005) at a rate of 100 nl/min with a 10-μl syringe fitted with a 34G bevelled needle by a microprocessor-controlled minipump (World Precision Instruments). Behavioral testing began 4 weeks after the rAAV injections.

Statistical Analyses

The statistical analyses were performed using ANOVA with Bonferroni post hoc comparison or unpaired Student's t tests. The significance level for all of the tests was set at $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2015.07.010.

AUTHOR CONTRIBUTIONS

Conceptualization, T.S. K.B, A.d.B. and D.v.C.; Methodology, T.S.; Investigation, T.S., H.-W.-C., D.K.T., F.I., C.N., M.S.; Resources, M.I., D.K.T., K.A.J.;

Writing-Original Draft, T.S. and K.B.; Writing-Review and Editing, T.S., K.B. and D.v.C.; Funding Acquisition, K.B. and D.v.C.; Supervision, K.B. and D.v.C.

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